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Nitrogen assimilation of Sterigmatocystis nigra and the effect of chemical stimulation *

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Inasmuch as the question of the relation of unstimulated and stimulated crops of *Sterigmatocystis nigra* to their carbohydrate supply had been several times investigated by Professor Richards and his students, it was decided to look into the effect of chemical stimulation upon the nitrogen consumption. Upon examination, it was found that this led largely to a study of the question of nitrogen fixation by the fungus under varying conditions, with the results given below. It will be remembered that Richards † determined the optimum stimulation for various salts; that Watterson ‡ worked over the effect of such stimulation on the CO_2 given off; that Richards § also determined the effect of irritation on the relative sugar consumption; and the present writer || worked along the same line and also considered the amount of oxalic acid excreted. This present paper is offered as a contribution to our knowledge of the nitrogen metabolism of *Sterigmatocystis nigra*. It has been carried out in the Botanical Laboratories

* The investigations here reported upon have been aided by a grant made to H. M. Richards from the Esther Herrman Fund of the New York Academy of Sciences.

† Richards, H. M. Die Beeinflussung des Wachsthums einiger Pilze durch chemische Reize. Jahrb. Wiss. Bot. **30**: 665. 1897.

‡ Watterson, A. The effect of chemical irritation on the respiration of fungi. Bull. Torrey Club **31**: 291. 1904.

§ Richards, H. M. The effect of chemical irritation on the economic coefficient of sugar. Bull. Torrey Club **26**: 463. 1899.

|| Latham, M. E. Stimulation of *Sterigmatocystis* by chloroform. Bull. Torrey Club **32**: 337. 1905.

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of Barnard College with the aid and advice of Professor Richards, to whom the writer is pleased once more to be able to acknowledge indebtedness and gratitude. A grant from the Esther Herrman Fund supplied some of the special apparatus used in prosecuting the work.

In 1895 Puriewitsch * published the results of an investigation on the nitrogen-fixing power of *Aspergillus niger* (*Sterigmatocystis*) and *Penicillium glaucum*, concluding that both organisms are able to bring uncombined nitrogen into chemical union, the amount so combined being in direct proportion to sugar supply and bearing no definite relation to the dry weight of the fungal felt. This work has been brought to question by various commentators † because of the small amount of nitrogen determined in analyses and the large chance of error inherent in the Kjeldahl method. Puriewitsch ‡ in his cultures used phosphoric acid in minute quantity to inhibit the growth of any nitrogen-fixing bacteria, not being sure, it seems, of his stock of the fungus. It has been indicated by the results that follow that the presence of this phosphoric acid will account for the small amount of nitrogen combined by the fungus, since the mycelium was stimulated by the acid and the ability to fix atmospheric nitrogen at the same time diminished.

Saida § has recorded a gain in total nitrogen in compound when working with *Aspergillus;* Winogradsky || had none. Berthelot ¶ had written in 1893 that he found a fixation of nitrogen by *Aspergillus niger*, *Alternaria tenuis*, and *Gymnoascus;* but of these only the *Alternaria* culture was pure and there the gain reached as high as 98 per cent. of the original nitrogen content. Brefeld ** investigated the action of *Ustilago*, which he found did not assimilate free

* Puriewitsch, K. Ueber die Stickstoffassimilation bei den Schimmelpilzen. Ber. Deuts. Bot. Gesells. **13**: 342. 1895.

† Lafar, F. Handbuch der Technischen Mykologie **3**: 1–69. 1904–1906.
Czapek, F. Biochemie der Pflanzen **2**: 125. 1905.
Jost, L. Plant Physiology, 235. 1907. [English translation.]

‡ Puriewitsch, K. *Loc. cit.*

§ Saida, K. Ueber die Assimilation freien Stickstoffs durch Schimmelpilze. Ber. Deuts. Bot. Gesells. **19**: 107. 1901.

|| See Lafar, F. Handbuch der Technischen Mykologie **3**: 11.

¶ Berthelot, M. Recherches nouvelles sur les microrganismes fixateurs de l'azote. Comptes Rendus **116**: 842. 1893.

** Brefeld, O. Versuche über die Stickstoffaufnahme bei den Pflanzen. Centralb. für Bakt. II. **8**: 24. 1902.

nitrogen and, furthermore, had no part in aiding the action of the host. In the case of *Phoma*, Saida * confirms Puriewitsch's conclusions with regard to the proportion between the amount of nitrogen brought into combination and the sugar at hand; and moreover, he records that when the fungus is growing on a solution poor in nitrogen compounds, then the CO_2 evolved becomes greater, that is, a greater expenditure of energy is indicated. Ternetz † found a gain in combined nitrogen due to the activity of a fungus isolated from the roots of *Oxycoccus*.

This summary of the work done on the relation of fungi to nitrogen-fixing is intended to touch the main points only. The literature may be had in detail from the works of Czapek ‡ and Lafar § and we are therefore content with this brief citation. The same question with bacteria has, of course, been more widely worked over, but with it we have made no attempt to deal.

In our former work, gaseous chloroform was the reagent used as a stimulant, but in this case we had recourse to zinc sulphate in solution, a stimulant with which Richards || had earlier worked. This was both because the difficulties in manipulation with the gas added to those of Kjeldahl analysis would probably have taken more time than we could hope to give to the work, and also because zinc sulphate has been more often used as a stimulant and its action is better known. The principle in each case is the same so that results would be similar. Aside from the difference in stimulant, the same methods were used as previously, the same superior reagents and insoluble glass, and the same care in preparation. A careful plate culture was prepared with the nutrient media in the gelatine to test the purity of the *Sterigmatocystis*. This showed no trace of bacteria whatever, so it was concluded that the spores were bacteria-free or at worst so little contaminated as to be highly satisfactory for our work. The growing cultures were kept under sealed bell-jars in order to exclude influences other than the one under observation, since we had found that the fungus is very sen-

* Saida, K. *Loc. cit.*

† Ternetz, C. Assimilation des atmosphärischen Stickstoffs durch einen torfbewohnenden Pilz. Ber. Deuts. Bot. Gesells. **22**: 267. 1904.

‡ Czapek, F. *Loc. cit.*

§ Lafar, F. *Loc. cit.*

|| Richards, H. M. *Loc. cit.*

sitive to traces of gases in the atmosphere. The recipe of the culture solution is this:

1.00 gm. NH_4NO_3	5.00 gm. sugar
0.50 " KH_2PO_4	100 c.c. water
0.25 " $MgSO_4$	trace of iron

The solution was made up in bulk in carefully cleaned and sterilized "non-sol" glass, boiled, cooled, and a suitable quantity taken at once for determining the nitrogen content. The rest was sown with spores, and zinc sulphate added in measured quantities where desired. The cultures were then allowed to grow for five or six days to reach a proper state, when they were reaped, the substratum analyzed at once for nitrogen, and the felts washed and dried preparatory to their analyses. In making all measurements, accurate pipettes and burettes were used, and for weighing the dry felt, a good Becker balance. To ascertain the nitrogen present the Gunning-Jodlbauer modification of the Kjeldahl process was followed. Two felts were cultivated at each stimulation and two determinations of nitrogen were made on each solution and on each felt. The procedure was to analyze the solution as provided to the fungus and again immediately after the crop was reaped, any difference being attributed to the action of the fungus. Then the dried felt was analyzed and the results obtained here added to the others and thus the full amount of nitrogen present in chemical union was determined. The most marked change is visible in the fluid substratum, because *Sterigmatocystis*, like nitrogen-fixing bacteria, may under favorable conditions excrete as a waste product some of the nitrogen compounds which it has formed from gaseous atmospheric nitrogen. The amount to be found in the make-up of the fungus itself being, as we find, relatively the same under all circumstances.

From six series grown after methods had been mastered, it was seen that (1) *Sterigmatocystis* grown normally does fix free nitrogen which is found to be in combination if nitrogen compounds be supplied in favorable quantity; (2) the amount of nitrogen so combined decreases if the culture be subjected to stimulation, both absolutely and relatively, *i. e.*, both in absolute quantity and in amount per gram of dry weight of crop produced, the diminution being shown most markedly by the decrease in combined nitrogen

in the substratum, where the amount of nitrogen fixed may become negative, that is to say, from which nitrogen may be used in metabolism; (3) the amount of nitrogen entering into the composition of the felt remains relatively the same in normal and stimulated growths; (4) the optimal stimulation from a carbohydrate point of view is not marked in nitrogen fixation where there is a gradual decrease in the amount caused to combine. These six series are wholly consistent within themselves.

TABLE I

Series.	Stimulation in terms of normal $ZnSO_4$ solution	Net dry weight Mg.	A Nitrogen furnished each culture Mg.	B Total nitrogen in each culture solution after growth Mg.	B—A Nitrogen fixed in each culture solution Mg.	Mg. of nitrogen fixed in solution per gram of felt	C Nitrogen in felt Mg.	Mg. of nitrogen per gram of dry felt	B+C Total nitrogen in felt and solution Mg.	B+C—A Total nitrogen fixed Mg.	Total nitrogen fixed in Mg. per gm. of dry felt
1	0.0000	200.2	115.4	175.8	60.4	301.5	11.1	55.5	186.9	71.5	357.2
	.0005	740.8		116.3	0.9	1.2	45.8	61.8	162.1	46.7	63.04
	.00075	722.6		112.2	—3.2	—4.5	69.3	63.5	181.5	66.1	91.47
	.001	767.5		112.6	—5.6	—7.4	44.9	58.5	157.5	42.1	54.85
2	0.0000	302.8	117.7	311.3	193.6	639.4	11.5	38.0	322.8	205.1	677.3
	.0003	740.5		118.4	.7	1.0	45.1	61.0	163.5	45.8	61.85
	.0005	705.5		144.7	27.0	38.3	44.2	62.6	189.1	71.4	101.20
	.0007	630.0		130.7	13.0	20.6	40.0	63.5	170.7	53.0	84.14
3	0.0000	185.0	141.6	132.8	—8.8	—47.6	10.4	56.1	143.2	1.6	8.65
	.0003	751.0		158.5	16.9	22.5	43.0	57.2	201.5	59.9	79.76
	.0005	705.0		150.0	8.4	11.9	43.8	62.2	193.8	52.2	74.04
	.0007	698.9		148.5	6.9	9.9	43.7	62.5	192.2	50.6	72.40
4	0.0000	270.0	155.1	185.2	30.1	111.5	14.9	55.4	200.1	45.0	166.8
	.0003	763.3		139.4	—15.7	—20.6	45.3	59.4	184.7	29.6	38.78
	.0005	773.0		146.9	— 8.2	—10.6	45.0	58.2	191.9	36.8	47.61
	.0007	772.4		129.4	—25.7	—33.3	40.3	52.1	169.7	14.6	18.90
5	0.0000	420.9	156.3	173.9	17.6	41.8	20.4	48.4	194.3	38.0	90.28
	.0005	728.9		146.8	— 9.5	—13.0	44.1	60.4	190.9	34.6	47.46
	.001	685.2		147.0	— 9.4	—13.7	35.0	51.1	182.0	25.7	37.51
	.0015	662.5		120.2	—36.1	—54.5	40.0	60.3	160.2	3.9	5.89
6	0.0000	316.5	160.3	115.4	—44.9	—141.9	11.6	36.6	127.0	—33.3	—105.2
	.0003	769.7		79.8	—80.5	—104.6	46.5	60.3	126.3	—34.0	—44.17
	.0005	853.7		65.8	—94.5	—110.7	52.0	60.3	117.8	—42.5	—49.78
	.0007	765.5		72.9	—87.4	—116.8	47.3	61.8	120.2	—40.1	—52.38

It may be seen by reference to Table I that with the control crops, those under normal conditions and without stimulation, there was found an increase of nitrogen over that of the initial

amount of nitrate that varied from 205.1 milligrams to 1.6 mg.; the amount supplied varying at the same time from 115.4 mg. to 156.3 mg. When the supply passed beyond the optimum point, when in this case 160.3 mg. were given the fungus at the outset, nitrogen was consumed. This would seem to indicate that the critical point with regard to nitrogen supply is slightly below 160.3 mg. in 50 c.c. of solution, although no attempt was made to determine the critical point with accuracy. It is known in the case of other nitrogen-fixing organisms that with an increasing nitrogen supply, the ability to utilize free nitrogen becomes less and less. At the point where it ceases, nitrogen must, of course, be consumed in the growth of the fungus. It will be noted how great is the difference between the amount of nitrogen fixed by the series just below the one in question and those with a much less amount supplied in the culture fluid. As the curve of nitrogen supply rises the curve of nitrogen-fixing ability falls, until the two cross at a point at which nitrogen consumption will begin. It may very well be that along such lines lies the inability of *Azotobacter* to fix nitrogen, ascribed by Beijerinck * to unexplained internal causes.

The average of the normals of all six series is 54.7 mg. or 199 mg. per gram of dry weight of the fungus felt; excluding from the average the series in which nitrogen was consumed, that is, series 6, the average becomes 72.2 mg. of nitrogen fixed, which is equivalent to 260.1 mg. per gram weight of dry crop. This union of atmospheric nitrogen diminishes with stimulation, being fairly constant around the point of optimal stimulation —.0005N $ZnSO_4$ — but finally disappearing. With a .0005N solution of $ZnSO_4$, the amount of nitrogen brought into combination varies from 34.6 mg. to 71.4 mg., the average being 48.3 mg. and this is 66.6 mg. per gram of dry felt produced. The effect of chemical stimulation upon nitrogen assimilation is therefore obvious at a glance if we compare normal and stimulated results, 72 mg. or 260.1 mg. per gram of dry felt in the unstimulated as against 33.2 mg. or 66.6 mg. per gram of dry substance in the stimulated. And again to compare the highest figures obtained at each point, a total gain of 174.2 per cent. of nitrogen over the amount supplied the normal

* Beijerinck, W. Centralb. für Bakt. II. 7: 561. 1901.

crop *versus* 60.6 per cent. gain in the crop grown at the optimal stimulation.

As was said before, commentators in their reviews have made objection to the work on nitrogen combination by fungi on the ground that the amounts determined by the analysis were too

TABLE II

AVERAGES OF FIVE SERIES, SERIES 6 BEING OMITTED

Stimulation in terms of normal $ZnSO_4$ solution	B − A Total nitrogen fixed in each culture solution Mg.	Mg. of nitrogen fixed in each solution per gram of felt	C Nitrogen in felt Mg.	Mg. of nitrogen per gm. of dry felt	B + C Total nitrogen in felt and solution Mg.	B + C − A Total nitrogen fixed Mg.	Total nitrogen fixed in Mg. per gram of dry felt
.0000	58.6	209.3	13.7	50.7	209.5	72.2	260.1
.0003	.6	1.0	44.5	59.2	183.2	45.1	60.2
.0005	3.7	5.6	44.6	61.0	185.6	48.3	66.7
.0007	— 2.2	— 1.8	48.3	60.4	178.5	46.1	66.7
.0010	— 7.5	—10.5	39.9	54.8	169.8	33.9	46.2
.0015	—36.1	—54.5	40.0	60.3	160.2	3.9	5.9

TABLE III

GRAND AVERAGES OF ALL SERIES

Stimulation in terms of normal $ZnSO_4$ solution	B − A Total nitrogen fixed in each culture solution Mg.	Mg. of nitrogen fixed in each solution per gram of felt	C Nitrogen in felt Mg.	Mg. of nitrogen per gm. of dry felt	B + C Total nitrogen in felt and solution Mg.	B + C − A Total nitrogen fixed Mg.	Total nitrogen fixed in Mg. per gram of dry felt
0.0000	41.3	150.8	13.3	48.3	195.7	54.7	199.2
.0003	—19.7	—25.4	45.0	59.5	169.0	25.3	34.1
.0005	—12.7	—13.8	45.8	60.9	174.3	33.2	47.3
.0007	—19.3	— 24.8	48.1	60.7	166.9	28.8	42.9
.0010	— 7.5	—10.5	39.9	54.8	169.8	33.9	46.2
.0015	—36.1	—54.5	40.0	60.3	160.2	3.9	5.9

small to be due positively to anything but experimental error. Now Puriewitsch's largest analysis gave 18.2 mg. of nitrogen with 9.8 mg. supplied at first, making a gain in fixed nitrogen of 8.4 mg. Saida's largest gain was 1.7742 mg. of nitrogen for *Aspergillus* and 10.536 mg. with a culture of *Phoma* which was

grown on a decoction of beet sugar, sugar, and some nitrogen source; while the gain for *Mucor* was 2.0699 mg. Ternetz in work with the "*Oxycoccus*-Pilz" found a gain of 3.2994 mg. of nitrogen. These are the more recent researches on the subject. In 1893, Berthelot wrote that in working with *Aspergillus* he had determined a gain of 26 per cent. in a certain culture, the crop giving an absolute gain of 7 mg. The figures we have been able to obtain have shown as high as 205 mg. in one instance, the lowest amount being 1.6 mg., but the total average is 72.2 mg. for five control cultures and 48.3 mg. for cultures at the optimal stimulation. These numbers in nitrogen analysis are not open to the objection made by the critics cited above, and by others. They are indeed rather large for nitrogen determinations. Of course they include all the nitrogen in the culture, both that excreted into the nutrient solution, and that entering into the make-up of the vegetative body as well as the original supply. The largest amount gained in solution was 193.6 mg. over and above the 117.7 mg. supplied.

Proofs of accuracy in the operations may be adduced from the facts that examinations of different specimens of a felt made at times at some interval from one another gave results which varied by only a small fraction of a milligram; and examinations of the reagents at different times were fortunate in the same respect. Again, the different results constantly obtained with normal and stimulated crops, all at the same time consistent within themselves, would seem to give further indication of some success in manipulation. So that on the whole, it is hoped that this work will be considered as having measurably confirmed the results of the earlier workers with normal cultures; and also as having brought forward some data of value, namely, that while stimulated crops behave more economically with regard to their carbohydrate consumption, and while the amount of nitrogen entering into the composition of the fungal felt is relatively the same as for a normal growth, yet with regard to their nitrogen relation these stimulated cultures are less thrifty than normal ones, which, unless the nitrogen supplied them be in too great quantity, are able to use gaseous nitrogen and to bring it into chemical combination even in excess of actual need.

To explain the reason for the activity of the organism along these lines, there are these suggestions: one, that the fixation of free nitrogen and its excretion in combined form may be a function connected with fructification, since stimulated felts do not produce spores; another, which is more theoretical and yet more probable, is that the stimulated crop, driven to its most rapid metabolic activity by the stimulant, is forced to consume its carbohydrate more economically and therefore finds less energy to use in effecting the combination of the relatively inert and difficultly combinable nitrogen and so must use the more readily assimilable compound nitrogen; or again, it may be that since by the presence of the stimulant, the fungus can consume carbohydrate more thoroughly and with less waste, therefore it finds, in what would be a normal amount under ordinary circumstances, a more than necessary amount under the favoring influence of the stimulant, which would of course be then potentially a too great supply and the result would be over-feeding in this direction and therefore there would be a tendency to lessened activity in expending energy for nitrogen combination. This last hypothesis is in accord with Moore's * conclusions on the activity of the root-tubercle bacteria in fixing nitrogen when well supplied with nitrogen compounds, but not in accord with the results of those who find that the fixation of nitrogen is directly proportional to the amount of sugar at hand.

If, however, the absolute numerical results be questioned, there can surely be no doubt of the fact that stimulation serves to decrease the amount of nitrogen to be found in the nutrient substratum, while it has little or no effect upon the relative amount combined in the fungal felt. And lastly, if even these results be questioned on the ground that the *Sterigmatocystis* was not pure but mixed with a nitrogen-combining bacterium — which we consider to have been refuted by the evidence of the plate culture before mentioned — there remains still the fact that in the body of the fungus hyphae in which the normal and stimulated fungi vary so widely in appearance, the relative nitrogen content of the chemical composition remains the same.

* Moore, G. T. Soil inoculation for legumes. U. S. Dept. Agr. Bur. Pl. Ind. Bull. 71. 1905.

The conclusions, summarized, are given below:

1. The work of Puriewitsch and others who found fixation of free nitrogen by *Sterigmatocystis nigra* (*Aspergillus niger*) is confirmed.

2. The fixation of free nitrogen is lessened and finally inhibited by the presence of zinc sulphate in minute quantities.

3. The relative amount of nitrogen entering into the substance of the fungal felt remains the same for stimulated and unstimulated cultures, the differences of behavior toward nitrogen appearing in the fluid substratum.

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